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Xenotransplante directo de cancro da bexiga em ratinhos imunodeficientes

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requesitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica do Professor Doutor Lúcio José de Lara Santos, Professor Auxiliar Convidado do Instituto de Ciências Biomédicas Abel Salazar e co-orientação da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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Resumo

A cistectomia radical é o tratamento standard do carcinoma urotelial invasivo da bexiga, contudo, cerca de metade dos paciente apresentam recidivas após a cirurgia e necessidade de quimioterapia sistémica. Pacientes com tumores invasivos da bexiga com características idênticas apresentam variações significativas na evolução natural da doença e resposta ao tratamento, reflectindo a composição heterogénea do tumor e a necessidade de uma tratamento personalizado. A avaliação prévia da sensibilidade do tumor à quimioterapia é especialmente importante e justificável em pacientes com elevado risco de apresentar resistência ao tratamento. Neste projecto, este risco é avaliado através da análise da expressão de marcadores moleculares tais como o CD147, previamente associado a mau prognóstico e resistência à cisplatina.

O principal objectivo deste projecto era estabelecer um modelo directo de carcinoma urotelial invasivo da bexiga humano através de xenotransplante em ratinhos imunodeficientes, caracterizar o modelo e avaliar a sua viabilidade como plataforma para o estudo da sensibilidade e resistência dos tumores à quimioterapia.

Um dos 9 fragmentos transplantados cresceu como implante primário nos ratinhos, tendo sido transferido com sucesso para novos animais, onde desenvolveu tumor em 2 dos 3 animais transplantados. A análise histológica e immuno-histoquímica (CD147, p53, p63, ki-67 e CK20) do xenotransplante, mostrou haver preservação da morfologia e fenótipo do tumor primário, pelo menos durante o estabelecimento do xenotransplante.

Estes resultados preliminares suportam o valor deste modelo para ensaios com fármacos, porém, são necessários estudos adicionais para validar o modelo e determinar o perfil dos pacientes que podem beneficiar desta abordagem.

Abstract

Radical cystectomy is a standard treatment for invasive bladder cancer, however, approximately half of the patients have disease recurrence after surgery and require systemic chemotherapy. Significant variations in the natural history and responses to treatment of patients with invasive bladder cancer are seen between tumors with identical features, reflecting the heterogeneity of the constituent tumor cells and the necessity of a personalized management approach. The assessment of tumor sensitivity to chemotherapeutic drugs is especially important and justifiable for patients with higher risk of showing drug resistance. In this project this risk was evaluated through the expression of molecular markers, such as CD147, that have been associated with poor outcome and cisplatin resistance.

With this project we aimed to establish an urothelial cancer xenograft model in nude mice from a sample of invasive urothelial carcinoma, characterize it, and assess the feasibility of this model for chemotherapy sensitivity and resistance testing.

1 of 9 specimens has grown as primary implant in nude mice and the xenograft generated was successfully transfered to other mice with a take rate of 2 in 3. Histologic and immunohistochemical (CD147,p53, p63, ki-67 and CK20) analysis showed that xenografts retain the morphology and phenotype of the original tumor, at least during xenograft establishment.

These preliminary results supports the value of this model for drug testing, however future studies are required to validate the model and determine the profile of the patients that may benefit for this approach.

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List of Acronyms

AA Amino Acid

AJCC American Joint Committee on Cancer

Ara-C Cytosine Arabinoside

CIS carcinoma *in situ*

CK20 Cytokeratin 20

dCK Deoxycytidine Kinase

dCTP deoxycytidine 5-triphosphate

DT Doubling Time

EMMPTIN Extracellular matrix metalloproteinase inducer

FFPE Formalin-fixed, paraffin-embedded

GC Gemcitabine and Cisplatin

H&E Haematoxylin-Eosin

ICH immunohistochemistry

IG immunoglobulin

ISUP International Society of Urological Pathology

MAP Mitogen-Activated Protein

MMP Matrix Metalloproteinase

MT Membrane Type

MVAC Methotrexate, Vinblastine, Doxorubicin and Cisplatin

PI3-kinase Phosphoinositide 3-kinase

RB Retinoblastoma-associated

s.c. Subcutaneously

TNM Tumor-Nodule-Metastasis

VEGF-A Vascular Endothelial Growth Factor-A

TSP-1 thrombospondin-1

TUR transurethral resection

UC Urothelial carcinoma

UICC International Union Against Cancer

VEGF vascular endothelial growth factor

WHO World Health Organization

Chapter 1

Introduction

1.1 Principles of tumor biology

A fully evolved tumor is a population of abnormal cells characterized by temporally unrestricted growth and the ability to grow in at least three different tissue compartments: the original compartment, the mesenchyme of the primary site (tumor invasion), and a distant mesenchyme (tumor metastasis) [16].

Tumorigenesis in humans is a multistep process involving genetic alterations that drive the progressive transformation of normal cells into highly malignant derivates [37]. The process through which normal cells evolve progressively to a neoplasic state results in specific lesions characteristic of the intermediate steps that can be identified by pathological analysis of the organ sites [28]. However, tumors are more than insular masses of proliferating cancer cells, they are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another. Normal cells, which form tumor-associated stroma, are participants in tumorigenesis contributing to the development and expression of certain abnormal abilities [38]. As the cancer progresses, the surrounding microenvironment co-evolves into an activated state through continuous paracrine communication, thus creating a dynamic signaling circuitry that promotes cancer initiation and growth, and ultimately leads to a fatal disease [86].

There are six main alterations in cells physiology that collectively enable tumor growth and metastatic dissemination (fig.1.1): 1. self-sufficiency in growth signals; 2. insensitivity to growth-inhibitory (antigrowth) signals; 3. evasion of programmed cell death (apoptosis); 4. limitless replicative potential; 5. sustained angiogenesis; 6. tissue invasion and metastasis [37]. The timing and sequence of these hallmark events can vary widely between tumors of the same type and within the same tumor, but ultimately, all these hallmark capabilities will be reached [37].

Self sufficiency in growth signals is characterized by a greatly reduced dependence on exogenous growth stimulation and sustains chronic proliferation [38]. Normal tissues control the production and release of growth-promoting signals required to progress from a quiescent to active state (cell growth and division cycle), thereby ensuring a homeostasis

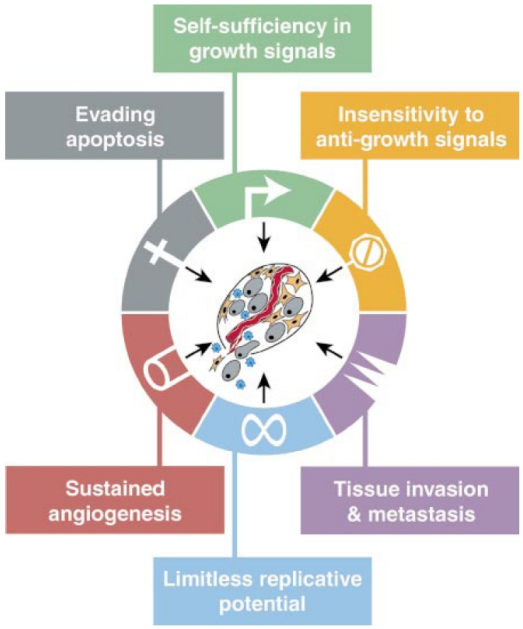

Figure 1.1: Acquired capabilities of cancer [37].

of cell number and thus maintenance of normal tissue architecture and function. Cancer acquires the capability to sustain proliferative signaling in a number of alternative ways: by the generation of their own growth signals, alteration of transcellular tranducers of these signals (number and/or activity), or alteration of intracellular circuits that translate those signals [37]. Alternatively, Cancer cells may send signals to stimulate normal cells within the supporting tumor-associated stroma, which reciprocate by supplying the cancer cells with various growth factors [6]. DNA sequencing analysis of cancer cell genomes have revealed somatic mutations that predict constitutive activation of signaling circuits usually triggered by activated growth receptors. Activating mutations afecting the stucture of the B-Raf protein, resulting in constitutive signaling to mitogen-activated protein (MAP) kinase pathway and mutations in the catalytic subunit of phosphoinositide 3-kinase (PI3-kinase) isoforms, which serve to hyperactivate the PI3-kinase signaling circuitry, have been detected in an array of tumor types [51, 19].

Insensitivity to antiproliferative signals permits the cancer cells to avoid quiescence or postmitotic states [37]. Antiproliferative signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surface of nearby cells [37]. Dozens of tumor suppressors genes that operate in various ways to limit cell growth and proliferation have been discovered through their characteristic inactivation in one or another form of animal or human cancer. The genes that encode RB (retinoblastoma-associated) proteins are key regulators of circuits that govern the decisions of cells to proliferate or, alternatively, activate senescence [38]. RB protein integrates

growth-inhibitory signals from diverse extracellular and intracellular sources and decides whether or not a cell should proceed through its growth-and-division cycle or commit apoptosis [38]. Additionally, several mechanisms of cell-to-cell contact inhibition that ensure normal tissue homeostasis may also be abrogated during the course of tumorigenesis. The product NF2 gene, cell-surface adhesion molecules and LKB1 epithelial polarity protein are some of the molecules involved in these mechanisms that may be altered in tumor cells [38].

Programmed cell death by apoptosis serves as a natural barrier to cancer development and the ability to evade apoptotic programs allows cancer cells to expand in number. The apoptotic pathway comprises a complex arrangement of sensors, effectors and regulators [66]. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality such extracellular survival factors, death signals, DNA damage and hypoxia [66]. Many signals that elicit apoptosis converge on the mitochondria, which responds to pro-apoptotic signals by releasing cytochrome C and ultimately activate intracellular proteases (caspases) that will execute the death program [37]. The evasion to apoptosis during carcinogenesis is often acquired through mutation on bcl-2 family of proteins that regulate the programmed cell death and mutations of p53, a tumor suppressor protein [66]. The protein p53 receives inputs from stress and abnormality sensors that function within the cell's intracellular operating systems and can stop cell-cycle progression until conditions have been normalized or trigger apoptosis, depending on the context.

The limitless replicative potential results from the gain of the abilities discussed above (growth signal autonomy, insensitivity to antigrowth signals and resistance to apoptosis). Since successive replications leads to the loss of protective telomerases resulting in chromosomal disarray and cell death, these processes alone do not ensure tumor growth [37]. However, upregulated expression of the enzyme telomerase, which adds telomeric DNA repeats to chromosome ends, allows malignant cells to maintain telomere length above a critical threshold and consequently unlimitless replication potential [18]. The presence of telomerase activity is correlated with a resistance to induction of both senescence and apoptosis.

Like normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by the process of angiogenesis, addresses these needs and is essential for a rapid clonal expansion of the malignant cells and to the development of a macroscopic tumor [37]. Angiogenesis is controlled by a balance of pro- and anti-angiogenic factors. Some of these angiogenic regulators are signaling proteins that bind to stimulatory or inhibitory cell-surface receptors displayed by vascular endothelial cells. The prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1), respectively [38]. Angiogenesis is induced during the multistage development of invasive cancers. Once angiogenesis has been activated, tumors exhibit diverse patterns of neovascularization [38]. VEGF gene expression can be upregulated both by hypoxia and by oncogene signaling and have been implicated in sustaining tumor angiogenesis during tumor development [38].

Tissue invasion and metastasis depends on all of the other five acquired hallmark capabilities, but the initial ability to uncouple from the primary tumor mass depends on the physical characteristics of the cells [37]. This step requires modulation of interactions with neighboring cells and the extracellular matrix, and production of proteases to degrade the extracellular matrix and basement membrane [37]. These alterations promote invasive behavior and metastasis, and are often associated to cadherins downregulation and overexpression of proteases like MMP2 and MMP9 [37]. The best characterized alteration involves the downregulation or mutational inactivation of E-cadherin, a key cell-to-cell adhesion molecule, in carcinoma cells [38].

1.2 Bladder cancer

1.2.1 Epidemiology

Bladder cancer is the 7th most common cancer worldwide, with an estimated 386,365 new cases (297,338 in men and 89,027 in women) and 150,165 deaths (112,255 in men and 37,910 in women) each year. [26]. Of the 1631 new cases of bladder cancer diagnosed in 2001 in Portugal, 46% invaded the muscularis propria and 15-30% of superficial bladder cancer progress to muscle invasion, usually within 5 years [48, 17]. The incidence of urinary bladder cancer increases with age and is considerably more common in males than in females, with an worldwide ratio of 3.4:1. In general, the prevalence this tumor in developed countries is approximately 6-times higher compared with that of developing countries [22].

The origin of bladder cancer is multifactorial, with tobacco smoking and occupational exposure to aromatic amines being the principal risk factors. Other etiological factors include analgesic abuse and chronic cystitis [22]. The influence of environmental factors in the development of bladder cancers may be explained by the exposure of the bladder epithelium to chemicals we ingest or their metabolites, especially when in high concentration or for extended periods of time.

Bladder neoplasms can arise from any of the bladder layers, being broadly classified as either epithelial or nonepithelial (mesenchymal), with over 95% being epithelial [89]. Epithelial tumors with differentiation toward normal urothelium are named urothelial or transitional cell carcinoma. Other primary epithelial tumors include adenocarcinoma, squamous cell carcinoma, and small-cell carcinoma, which comprise approximately 6, 2, and less than 1% of bladder tumors, respectively [35]. Neoplasms derived from mesenchymal tissue differentiate toward muscle, nerve, cartilage, fat, connective tissue and blood vessels.

1.2.2 Classification

The classification of urothelial neoplasms has been an area of significant controversy and debate over the last decade, with several classification/staging systems been proposed for these neoplasms. Pathological stage is the most important determinant of prognosis and treatment for bladder cancer [13]. An ideal staging system should accurately reflect

the natural history of cancer at this site, describe the total cancer burden, assess the extent of spread at the time of diagnosis, and stratify patients into prognostic groups for treatment planning. Adoption of an uniform staging system permits comparison of therapeutic interventions among different institutions.

Until a few years ago, the 1973 World Health Organization (WHO) scheme appeared to be the most widely recognized and utilized. In an attempt to provide a method of categorization that more accurately reflects a tumor's biologic behavior, and to establish greater uniformity in defining urothelial lesions, the WHO/International Society of Urological Pathology (WHO/ISUP) issued a consensus statement in 1998 regarding the classification of urothelial lesions.

Growth pattern

The growth pattern of the tumor may be determinated macroscopically or microscopically. There are four main patterns [78]:

(1) Papillary, in wich the tumor is growing into the lumen of the bladder;

(2) Infiltrating, in which it is growing into the wall of the bladder;

(3) Papillary and infiltrating, in which it is growing into the lumen and into the wall;

(4) Non-papillary and non-infiltrating, in which the tumor is confined to the surface, i.e., *in situ*.

This information is particularly important because of its relationship to prognosis. Papillary carcinomas have a much better prognosis than infiltrating carcinomas [78].

Histological grading

Several systems have been used to grade and classify bladder neoplasms. In 1972 the World Health Organization (WHO) proposed a system of grading (1-3) based on the degree of anaplasia of tumor cells (table1.1) [78, 35]. Grade 1 tumors have an orderly arrangement of normal cells lining papillae and minimal architectural abnormalities, anaplasia, or pleomorphism. Mitotic figures are rare or absent. Grade 3 tumors demonstrate extreme nuclear abnormalities, disordered architecture, loss of polarity, and frequent mitotic activity. Although the definitions of grade 1 and grade 3 tumors are explicit, grade 2 is a diagnosis of exclusion. In the early 1990s, several factors emerged that resulted in the need to reevaluate this approach. Efforts were made to formulate an evidence-based system that would provide stricter definitions for bladder tumor grading and have a larger effect on prognosis and clinical decision-making, leading to the publication of a consensus classification system for urothelial neoplasms by the WHO and the International Society of Urologic Pathologists (ISUP) in 1998 (table1.2) [23, 35]. The clinical significance of this schema was validated by subsequent studies, and in 2004 it was accepted as the standard classification schema [22]. According to this system, urothelial tumors can be divided in infiltrating urothelial carcinomas (UC) and non-invasive urothelial neoplasias based on the presence of tumor invasion into bladder wall, and classified as low-grade or high-grade based upon the degree of nuclear anaplasia and architectural abnormalities [22]. It is clas-

Table 1.1: 1973 WHO grading criteria

Grade 1	Tumors with the least degree of cellular anaplasia compatible with diagnosis of malignancy
Grade 2	Histologic features between grades 1 and 3
Grade 3	Tumors with the most severe degrees of cellular anaplasia

sified as infiltrating, when invades beyond the basement membrane [22]. The non-invasive urothelial neoplasias includes flat lesions with atypia, like carcinoma in situ, and papillary neoplasm (low grade and high grade).

Pathological staging

Another criterion for assessing the behavior of a tumor is to determine the depth of its infiltration into the bladder wall or adjacent tissue, also designated as staging. Tumors that have not invaded into muscularis propria are described as superficial bladder cancers, and includes noninvasive papillary urothelial carcinoma (pTa), carcinoma *in situ* (CIS) (pTis), and tumor invading into the lamina propria (pT1) (figure1.2) [12]. Stage pT2 and pT3 carcinomas are defined by tumor invasion into muscularis propria and into perivesical soft tissue, respectively [12].

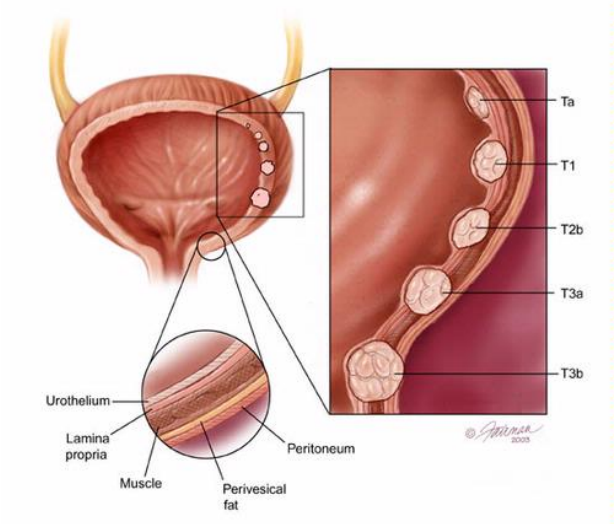

Figure 1.2: TNM staging based on anatomic extent of cancer.

The tumor-nodule-metastasis (TNM) classification is based on the anatomic extent of cancer, being helpful to plan treatment, assess prognosis, evaluate results of treatment and stratify patients for therapeutic studies [34].

Table 1.2: 2004 WHO/1998 ISUP classification of urothelial tumors [22].

Infiltrating urothelial carcinoma
With squamous differentiation
With glandular differentiation
With trophoblastic differentiation
Nested
Microcystic
Micropapillary
Lymphoepithelioma-like
Lymphoma-like
Plasmacytoid
Sarcomatoid
Giant cell
Undifferentiated
Non-invasive urothelial neoplasias
Urothelial carcinoma *in situ*
Non-invasive papillary urothelial carcinoma, high grade
Non-invasive papillary urothelial carcinoma, low grade
Non-invasive papillary urothelial neoplasm of low malignant potential
Urothelial papilloma
Inverted urothelial papilloma

The TNM system, originally described by denoix in 1952, is based on the premise that the choice of treatment and the chance of survival are related to the extent of the tumor at the primary site (T), the presence or absence of cancer cells in regional lymph nodes (N), and the presence or absence of metastasis beyond the regional lymph nodes (M) [34]. The first international TNM recommendations for the clinical stage classification of cancers of the breast and larynx were published by the International Union Against Cancer (UICC) in 1958 [34]. Between 1960 and 1967, 9 UICC brochures were published describing proposals for the classification of tumors at 23 body sites. In 1968, these brochures were combined, representing the First Edition of TNM. Over the years, variations in the rules of classification of certain sites were introduced, but in 1982, the national TNM committees agreed to formulate a single TNM classification. The Fourth Edition of TNM, coordinated with the UICC and American Joint Committee on Cancer (AJCC) and representing the achievement of a worldwide agreement for the staging of adult solid tumors was then published [47].

The TNM staging may be clinical (cTNM) or pathological (pTNM). The first is based on evidence acquired before treatment by physical examination, imaging, endoscopy or biopsy, whereas the pathological classification is based on evidence acquired before treatment, supplemented or modified by the additional evidence resulting from surgery and pathological examination. The pathological assessment requires adequate resection of the tumor (pT), removal of adequate number of regional nodes (pN), and microscopic examination to assess distant metastasis (pM) [33].

Table 1.3 describes the TNM classification of urinary bladder carcinomas. T is divided into 4 major parts (T1-T4), expressing increasing size or spread of primary tumor, whereas N and M comprises 2 categories each (0-1), indicating absence or presence of tumor. The stage of the cancer is defined by the combination of the TNM categories.

Table 1.3: TNM staging system for urinary bladder carcinomas, adapted from [22].

Primary Tumor	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary tumor
Tis	Carcinoma *in situ*
T1	Tumor invades lamina propria
T2	Tumor invades muscularis propria bladder wall
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically (extravesical mass)
T4	Tumor invades an adjacent organ
T4a	Tumor invades prostate, uterus or vagina
T4a	Tumor invades pelvic or abdominal wall
Regional Lymph Nodes (N)	
NX	Regional lymph node cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a simgle lymph node, $2cm$ or less in greatest dimension
N2	Metastasis in a simgle lymph node, $> 2cm$ but not $> 5cm$ in greatest dimension, or multiple lymph node, none $> 5cm$ in greatest dimention
N3	Metastasis in a lymph node $> 5cm$ in greatest dimension
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant Metastasis

1.3 Muscle invasive bladder cancer

The majority of patients with bladder cancers are diagnosed with a superficial, low-grade, and non-invasive disease. These patients are treated by local excision often followed by intravesical infusion of adjuvant therapy. Despite these treatments approximately 70% of patients with superficial disease have tumor recurrence with 10 to 20% ultimately progressing to invasive disease [17].

1.3.1 Cancer biology

In order to understand the biology of bladder cancer we need to have in consideration the unique context of the organ where it arises. The urothelium is a highly specialized epithelium, situated between the urine and the blood [56]. The normal urothelium acts as a permeability barrier protecting the underlying tissues from the toxic urinary substances and adjusting its surface area actively and reversibly during micturition cycles [81, 64]. Furthermore, the uroepithelium releases various mediators and neurotransmitters which may allow the epithelium to transmit information about the state of the mucosa and bladder lumen to the underlying nervous, connective and muscular tissue [4].

Histologically the urothelium is comprised of a single-cell type phenotypically distint in the different cell layers attributed to the different degree of cellular differentiation [111]. When viewed in cross section, the urothelium is composed of umbrella, intermediate, and basal cell layers [64, 56]. Umbrella cells are a single layer of highly differentiated and polarized cells, whose morphology and size depends on the filling state of the bladder [56]. These cells are polyhedral, and often multinucleated cells, thought to be derived from the intermediate cells via cell-cell fusion [64, 56]. The intermediate cells are often pyriform, mono-nucleated, and connected to one another and to the underlying umbrella cells by desmoses [53]. This cell population rapidly differentiates when the overlying umbrella cells are lost or removed. Cells in the basal layer are the smallest in size and least differentiated, and this is where the proliferative compartment and stem cells are believed to reside [62, 56]. These cells can differentiate to become intermediate cells whose layer thickness depends on species [56].

In developed countries, more than 90% of bladder cancer are transitional cell carcinomas of urothelial origin, followed by squamous cell carcinoma, adenocarcinoma and small cells carcinoma [21].

The histology of invasive urothelial carcinoma has no specific features, it shows infiltrating cohesive nests of cells with moderate to abundant amphophilic cytoplasm and large hypercromatic nuclei [22]. The nucleus is typically pleomorphic and has irregular contours. The nucleoli are highly variable in number and appearance, ranging from single or multiple of small size to large eosinophilic [22]. Mitotic figures are common, with numerous abnormal forms. The stoma often contains lymphocytic infiltrate with a variable number of plasma cells. Neutrophils and eosinophils are rarely prominent. Foci of squamous and glandular differentiation are also common. Divergent differentiation into squamous or glandular cells is frequently associated with high grade and stage UC [11].

1.3.2 Clinical features

The type and severity of clinical signs and symptoms of invasive UC depends on the extend and location of the tumor. The most common presenting symptom is painless gross hematuria that may be followed by clotting and painful micturition. These symptoms may also be associated with frequency, as in case of large tumor, or irritative symptoms like dysuria and urgency [22].

Direct cystoscopic examination visualization of the bladder and transurethral resection (TUR) continues to be the gold standard in the diagnosis of patients presenting symptoms of urothelial carcinoma [5]. The specimen obtained by TUR permits the assessment of tumor histology, grade and depth of invasion. The determination of size, and the presence of extravesical extension or invasion of adjacent organs should be performed in order to stage the disease [22, 5]. The tumor grade and stage are the primary prognostic variables critical for prediction of patient outcome and selection of appropriate therapy.

1.3.3 Bladder cancer biomarkers

Bladder cancers are a mixture of heterogeneous cell populations, and numerous factors are likely to be involved in dictating their recurrence, progression, and the patient's survival [57]. Prognostic markers play a key role in clinical practice, distinguishing patients into different risk groups and thus informing treatment strategies and aiding patient counselling.

A better understanding of the molecular mechanisms involved in carcinogenesis and cancer progression has identified a large number of molecular markers of bladder cancer, each of which have a potential diagnostic and prognostic value. To have clinical revelance, they must add some predictive capacity beyond that offered by the conventional and pathological paramenters.

To date, numerous potential markers have been identified by a variety of molecular biology and genetic studies. Molecular markers such as Ki-67 and TP53 do appear to have some promising correlations with bladder cancer development, agressiveness and progression. However, many of them have shown insufficient sensitivity and specificity in predicting the prognosis of bladder cancer patients, in particular, the high-risk patient groups that are at risk of progression and recurrence [57].

Emmprin

Extracellular matrix metalloproteinase inducer (emmprin) or CD147 is a highly glycosylated transmembrane protein that belongs to the immunoglobulin superfamily [8]. Emmprin is a tumor cell derived matrix metalloproteinase (MMP) inducer that is expressed on the tumor cell surface and stimulates nearby fibroblasts and endothelial cells to produce MMP, facilitating invasion by cancer cells [7, 8]. In addition to induction of MMP production, emmprin also mediates angiogenesis via stimulation of vascular endothelial growth factor (VEGF), anchorage-independent growth and multidrug resistance in a hyaluronan-dependent fashion [75, 116, 106]. Constitutive expression of emmprin at low levels exists

in most cell types, where it is involved in physiologic processes, moreover its expression at high levels occurs during remodeling processes such as inflammation, embryonic development and wound healing [79].

Structure

Emmprin consists of a 185 amino acid (aa) extracellular region containing two immunoglobulin (ig) domains, a 24 aa residue transmembrane domain and a 39 aa cytoplasmatic domain (fig.1.3) [8].

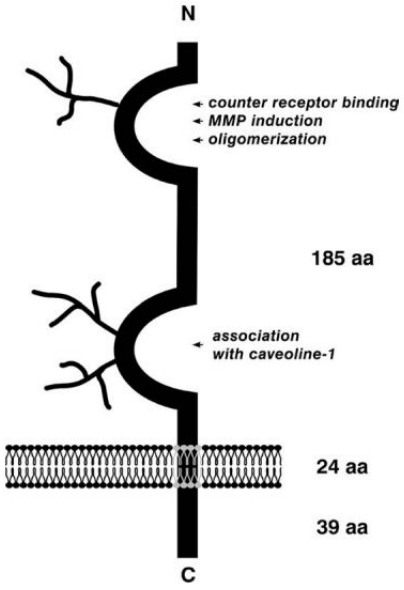

Figure 1.3: Scheme of emmprin molecule [31].

The single transmembrane sequence is completely conserved among species and the presence of a charge acidic residue (glutamic acid) within the hydrophobic sequence of this domain suggests that transmembrane associations with other membrane proteins are likely to occur [76].

The extracellular region contains three N-linked glycosylation sites that are variably glycosylated. The degree of glycosylation is regulated through interaction with caveolin-1 and determines emmprin activity and function [105]. Emmprin forms homo-oligomers in a cis-dependent manner in the plasma membrane. This process is mediated by the N-terminal Ig-like domain, that is essential and sufficient for oligomerization [117].

Function

To date, emmprin has been reported to induce several MMP, including collagenase (MMP1), gelatinase A (MMP2), stromelysin (MMP3), gelatinase B (MMP9), membrane type (MT) 1 MMP (MMP14) and MT2 MMP (MMP15) [94, 116]. In addition to production of MMPs, emmprin also binds MMP1 and retains it in the cell surface, promoting the

turnover of pericellular collagen [36].

The MMP-inducing function of emmprin in part involves the molecule acting as a counter receptor for itself in the N-terminal Ig domain [104]. Emmprin also interacts with integrins and caveolin-1. Over expression of caveolin-1 causes a decrease in clustering of emmprin on the cell surface and decrease induction of MMP1, contributing to the onco-suppressive effects of caveolin-1 [105]. Association with caveolin-1 also prevents formation of highly glycosylated forms of emmprin, inhibiting emmprin aggregation and activity [105].

Role of emmprin in cancer

Immunochemistry analysis showed that emmprin expression is significantly upregulated in bladder cancer when compared with normal urothelium [80]. Other studies identified emmprin overexpression as an independent predictor of worse prognosis in patients with bladder cancer, where it was significantly associated with poor patient survival and higher recurrence risk (Fig.1.4) [90, 2, 112]. Emmprin overexpression was also correlated with tumor stage, histologic grade, and lymph node status, suggesting that its expression might be important for the development of malignant potential in bladder cancer [112]. There are also evidences that the expression levels of emmprin in invasive urothelial cancer of the bladder are higher than those in noninvasive tumors [90].

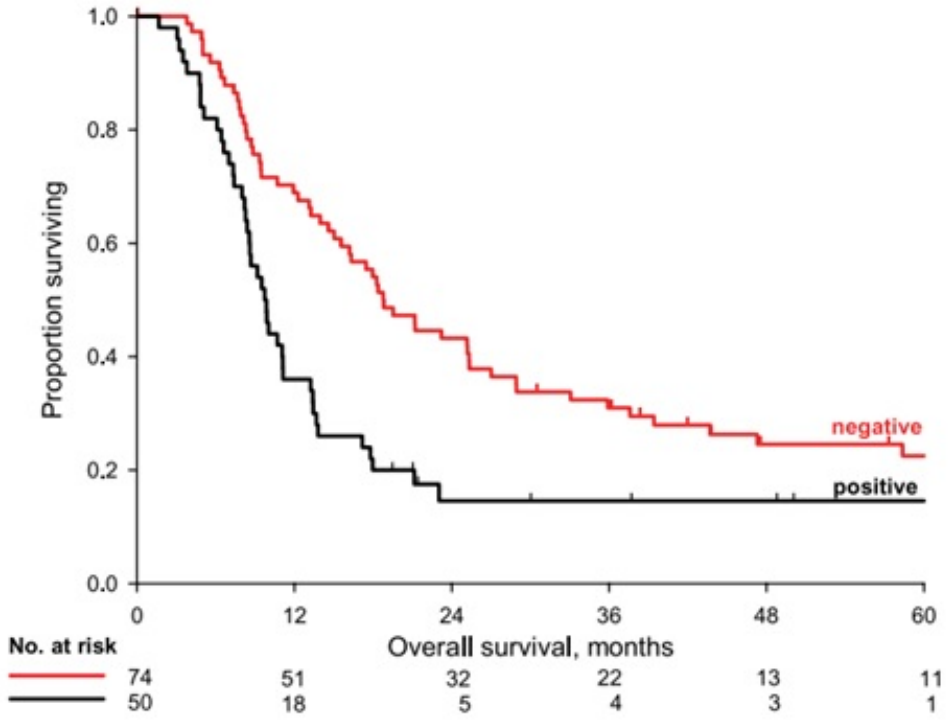

Figure 1.4: Overall survival of patients with urothelial carcinoma according to emmprin expression [2].

Emmprin might promote tumor invasion and metastasis through stimulation of MMPs and VEGF synthesis. Additionally, it also induces chemoresistance via the production of hyaluronan, and confers cancer cells resistance to anoikis [75, 116, 106, 115].

Emmprin in tumor cells stimulates fibroblasts, endothelial cells or cancer cells themselves, to secrete an increased amount of metalloproteinases (MMPs), which are responsible for the degradation of the extracellular matrix facilitating tumor invasion and metastasis [8]. A study showed that emmprin was the most frequently upregulated mRNA and protein in micrometastatic cells isolated from the bone marrow of cancer patients, indicating a key role of emmprin in the processes of tumorigenesis and metastasis [58].

Hyaluronan is a very high molecular weight polysaccharide, composed of repeating disaccharide units, and is associated with the pericellular matrix surrounding proliferating and motile cells in normal and pathological systems, where it serves both structural and signaling functions [108]. On tumor cells, the interaction of hyaluronan with receptors on the surface induces anchorage-independent growth and plays a critical role in cell survival and multidrug resistance, possibly by stimulating the PI3-kinase and MAP kinase cell survival pathways [75].

Anoikis is a form of apoptosis induced by loss or alteration of cell-cell or cell-matrix anchorage. This process was studied in breast carcinoma cells, where the expression of high levels of emmprin were associated with higher viability and resistance to anoikis [115]. However, there are no studies of the influence on emmprin expression in anoikis in bladder cancer cells.

p53

The p53 gene and protein both play critical roles in regulation of the cell cycle, apoptosis, and DNA synthesis and repair, with mutations in p53 thought to be one of the key central events in urothelial carcinogenesis [97, 24, 32]. Most mutations in P53 are missense and induce conformational changes in the p53 protein, causing an increase in the protein half-life, which is the basis of its nuclear accumulation that is detectable by immunohistochemistry (IHC) [25]. Thus, nuclear overexpression generally correlates with inactivation of P53.However, overexpression could also be the result of a physiological response to DNA damage [68]. Both p53 mutations and p53 nuclear accumulation have been associated with increased grade and stage of cancer, and poor clinical outcome [25].

p63

p63, a member of the p53 gene family located on chromosome 3q27–28, encodes multiple proteins that may either transactivate p53 responsive genes (TAp63) or act as a dominant-negative factor toward p53 and p73 (δ Np63) [113]. p63 is highly expressed in the basal or progenitor layers of many epithelial tissues, and it seems to play a key role in growth-suppression and differentiation of transitional epithelium [114, 109]. It is suggested that TAp63 isoform is required for initiation of epithelial stratification and maintains a proliferative potential of basal cell, while δNp63 allows cells to respond to signals required

for maturation. In a normal bladder, TAp63 is expressed in basal cells, whereas δNp63 is absent or weakly expressed in these cells [82]. In bladder carcinomas, elevated expression of δNp63 and decreased expression of TAp63 has been observed [82]. These results suggest that δNp63 might contribute to progression of bladder carcinomas. The diminished p63 expression in TCCs may represent the loss of differentiation-associated, and therefore, growth-inhibitory p63 isoforms, which may be associated with tumor stage and grade [109].

Ki-67

Ki-67 is a nuclear protein expressed by proliferating cells and can be used as a measure of biological aggressiveness of a malignancy [10]. Ki-67 is an established marker of cell proliferation, that can be observed immunohistochemically, being present during the G1, S, G2, and M stages of the cell cycle, but absent in resting cells (G0 phase) [10, 69]. In bladder TCC, increased Ki-67 expression is related to tumor grade and stage, and was independently associated with cancer recurrence and survival after radical cystectomy [69]. Ki-67 labelling index distinguishes low-grade from highgrade tumors. However, Ki-67 immunostaining can be most useful for patients with organ-confined disease (pT3 N0) undergoing cystectomy, where it is strongly associated with recurrence and bladder cancer-specific death, possibly suggesting the need for additional therapy [32].

CK20

Cytokeratin 20 (CK20) is a marker for tumors of low stage and grade, and is expressed in the umbrella cells of normal urothelium and reactive atypia, where it is detected by IHC [77]. When CK20 expression in bladder tumors is limited to the umbrella and superficial cells, tumours are typically of low grade with a mild disease course, while expression in the entire urothelium in more than 10% of the tumour cells is associated with higher tumour grade, and an increased risk of recurrence and progression [83, 14]. Aberrant CK20 expression thus appears to signify more aggressive disease.

1.3.4 Management of muscle-invasive bladder cancer

Radical cystectomy is the standard procedure therapy for patients with muscle-invasive urothelial carcinoma of the bladder [5]. This surgery consists of the removal of the bladder, prostate, seminal vesicles, and perivesical tissue in men, and removal of the bladder, uterus, fallopian tubes, and anterior vaginal wall in women. Lymphadenectomy, as part of definitive surgery, has become the standard care and plays a prognostic and possibly therapeutic role in this disease [100, 42]. Bladder sparing regimens may also be appropriate for patients who are medically unfit for surgery or seek an alternative [5].

Even with optimal surgical management, approximately 50% percent of patients with muscle-invasive disease relapse and die of their disease. Most patients who recur after cystectomy relapse with distant metastases and 30% have local recurrences [30]. The risk of

recurrence and bladder cancer related death increases significantly with increasing pathological tumor grade, nodal status, quality and extent of surgery performed and advancing final pathological stage [96].

Cisplatin based combination chemotherapy regimens like MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) or GC (gemcitabine and cisplatin) prolongs survival and are considered the standard chemotherapeutic regimens for patients with invasive carcinoma of the urothelium [5]. Both combinations are equally effective, although GC is less toxic. The overall response rate with these regimens ranges from 50 to 70%, with complete responses seen in 15 to 20% of cases [102, 107]. Patients unfit for cisplatina based chemotherapy may be palliated with carboplatin based regimen or single-agent taxane or gemcitabine. Vinflunine is an option for second line therapy in patients progressing with first line platinum based chemotherapy [5].

1.3.5 Chemotherapy for invasive bladder cancer

In patients with pT3-4 and/or N+M0 disease, 5-year survival after radical cystectomy is only 25 to 35% at best [41]. Optimal therapy to muscle-invasive bladder cancer aims to prevent local recurrence, decrease the probability of metastases and improve survival. Current evidence shows that this goals are best achieved by combining cisplatin based chemotherapy with high quality radical surgery.

MVAC was introduced in 1985 and became the first successful chemotherapy regimen used for metastatic bladder cancer [101]. Overall response proportion were achieved in 72% of patients, including complete responses in 36%. Because MVAC also produced significant responses in the primary tumor, the regime was given to patients before cystectomy as neoadjuvant chemotherapy and after, as adjuvant chemotherapy for muscle invasive bladder cancer [95].

Some randomized trials studying the role of the adjuvant therapy after cystectomy suggest survival benefit from chemotherapy with significant improvement in 3-years disease-free survival [103]. An adjuvant approach allows the selection of patients at highest risk for surgical failure based on accurate pathological staging in the cystectomy specimen rather than in transurethral biopsy. This selection avoids overtreating patients who are estimated to have a reasonable outcome from surgery alone.

Although MVAC is the established chemotherapy regimen, significant toxicity limits its operative use to the fittest patients. In patients with advanced or metastatic urothelial carcinoma, the combination of gencitabin plus cisplatina have comparable response proportions and similar overall long term survival when compared with MVAC but much less toxicity [110]. Reasons for not treating with adjuvant chemotherapy include poor performance status, frequent surgical complications, major comorbidities, psychological distress, poor renal function, old age and patient refusal [41].

1.4 Cisplatin

Cis-Diamminedichloroplatinum(II) (cisplatin or cis-DDP) is an inorganic complex formed by an atom of platinum surrounded by chlorine and ammonia atoms in the cis position of a horizontal plane (fig.1.5).

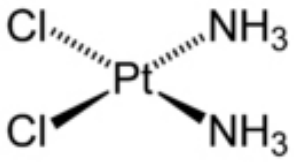

Figure 1.5: Cisplatin structure.

Cisplatin entered into clinical trials in the early 1970s and was found to have significant antitumor activity against testicular cancer, lymphoma, squamous cell carcinoma of the head and neck, ovarian cancer, and bladder cancer [46, 65, 45]. Because of its significant therapeutic effect in these tumors and activity against a number of other solid tumors, it became the most frequently used antitumor agent. Initially, it was used as a single agent in patients with metastatic disease refractory to chemotherapy and, subsequently, it was tested in nonrefractory disease. Cisplatin was also included in various combination regimens and employed following the failure of other, more conventional chemotherapies.

1.4.1 Mechanism of action

Cisplatin enters the cell by diffusion or active transport. Once inside the cell, its chloride ligands are replaced by water molecules generating a positively charged aquated species that can react with nucleophilic sites on intracellular macromolecules to form protein, RNA and DNA adducts [54]. Studies with Escherichia coli and eukaryotic cells deficient in DNA repair, showed that DNA adducts are the key toxic lesions formed by cisplatin [59, 29]. The reaction with DNA yields monofunctional adducts, intrastrand crosslinks and interstrand crosslinks with the platinum atom coordinated to the N7 position of guanine or adenine [20, 9, 119]. Adduct formation results in inhibition of DNA replication, RNA transcription, arrest at the G2 phase of the cell cycle, and/or programmed cell death [93, 15, 98, 72].

1.4.2 Resistance

Reduced intracellular accumulation of cisplatin, which may arise because of decreased uptake or increased efflux, is frequently observed in cisplatin resistant cells [3]. Another mechanisms of resistance is associated with the inactivation of cisplatin by sulfur-containing molecules mediated by the binding to glutathione molecules [71]. Cisplatin can be covalently linked to glutathione and this complex can be transported out of the cell by an

ATP-dependent pump [49]. Metallothioneins are a family of cysteine rich proteins involved in Zn2+ homeostasis and in the detoxification of heavy metals such as cadmium. Metallothioneins binds to cisplatin and may also affect sensitivity to this drug [85].

Alterations in the DNA repair status and in the expression of oncogenes (such as c-fos, c-myc, H-ras, c-jun, and c-abl) and tumor suppressor genes (such as p53) have also been implicated in the cellular resistance to cisplatin [54].

1.5 Gemcitabine

Gemcitabine (2',2'-difluoro 2'-deoxycytidine, dFdC) is an analogue of cytosine arabinoside (Ara-C) from which it differs structurally due to its fluorine substituents on position 2' of the furanose ring (Figure 1) [44].

Although ara-C and gemcitabine are structurally similar, there are major differences in their antitumor activity. Ara-C is one of the most effective agents for the treatment of adult acute myelogenous leukemia, but it does not show activity against solid tumors as does gemcitabine [55, 73].

Currently, gemcitabine is indicated for the treatment of bladder cancer, advanced non-small cell lung cancer, advanced pancreatic cancer, breast cancer and ovarian cancer.

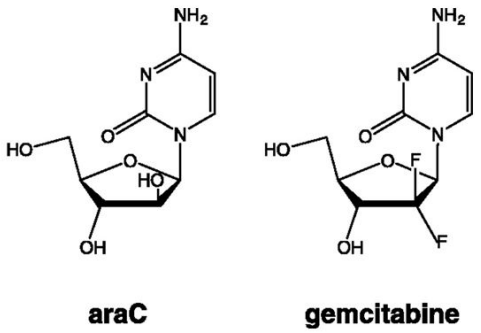

Figure 1.6: Ara-C and Gemcitabine chemical structure.

1.5.1 Mechanism of action

Gemcitabine is a prodrug which requires cellular uptake and intracellular phosphorylation. After entering the cell through a membrane nucleoside transporter, gemcitabine is phosphorylated to gemcitabine monophosphate by deoxycytidine kinase (dCK), which is then converted to gemcitabine di- and triphosphate [39, 67]. These are the active drug metabolites.

The cytotoxic effect of gemcitabine is attributed to a combination of the actions performed by these two metabolites. The active metabolite, gemcitabine triphosphate, com-

petes with the natural deoxycytidine 5-triphosphate (dCTP) for the incorporation into the DNA strand. Once incorporated, an additional natural nucleoside is added, masking gemcitabine and preventing DNA repair by base pair excision [87, 74]. Thereafter, the DNA polymerases are unable to proceed, a process designated as 'masked DNA chain termination', leading to growth inhibition or cell death.

The active diphosphate metabolite of gemcitabine also inhibits DNA synthesis indirectly through inhibition of ribonucleotide reductase RR [43, 74]. This effect blocks the *de novo* DNA synthesis pathway and self-potentiates gemcitabine activity by decreasing intracellular concentrations of normal deoxynucleotides triphosphate (particularly dCTP). Reduction in cellular dCTP results in increased gemcitabine nucleotide incorporation into DNA and increased formation of active gemcitabine di- and triphosphates, since dCK activity is down-regulated by high cellular dCTP levels [40, 73]. Other self-potentiating mechanisms of gemcitabine include a decreased elimination of gemcitabine nucleotides by direct inhibition of cytidine deaminase, an enzyme that inactivates gemcitabine [87].

Gemcitabine can also be incorporated into RNA and inhibit RNA synthesis. While incorporation of gemcitabine metabolites into RNA are concentration dependent, the extent of RNA synthesis inhibition seems to be cell-specific [91]. Gemcitabine exhibits cell phase specificity, being primarily active against cells undergoing DNA synthesis (S-phase) and under certain conditions blocking the progression of cells through the G1/S phase boundary.

1.6 Xenograftic model

In 1971, Povlsen and Rygaard demonstrated that nude mice could accept heterotransplantations of human adenocarcinomas of the colon and rectum and that these tumors could be transferred consecutively from mouse to mouse, producing adenocarcinomas identical with the original tumor tissues [88]. Since that time, the heterotransplantation of various human tumors into immunodepressed hosts has been used to generate tissue for the study of the biology, morphology and biochemical characteristics of human tumors [63, 61].

Xenografts and genetically engineered mice models have been used as the front line in predicting efficacy and finding toxicities for cancer chemotherapeutic agents before entering the clinic. While the development of genetically engineered mouse models has contributed greatly towards understanding the process of carcinogenesis and target selection, the xenograft models established by injecting cultured cancer cells subcutaneously in a nude/SCID mice, have been more popular for drug screening purposes due to its faster, low cost, and ease establishment and monitorization [84]. Orthotopic implantation of these cell lines recapitulates some of the features of the cancers from which such cells were derived, however many agents that show consistent and potent anticancer efficacy in xenografts, fail in the clinical trials due to lack of efficacy [52]. This might be due to reliance of xenografts on small numbers of homogeneous cell lines adapted to the artificial culture conditions and acquisition of biological characteristics significantly different from the original natural clone over serial passages in culture. On the other hand, the establishment

of direct xenografts of human cancers allows the transplantation of all cellular fractions of the tumor, maintaining the original cell heterogeneity, tumor phenotype and malignant potential. This approach has demonstrated superior correlation of chemosensitivity and specificity data for individualized therapy [84]. Studies where intact tissue from the patient was transplanted into the mice have shown the excellent patient response prediction rates of 90% and 97% for chemosensitivity and chemoresistance, respectively [27]. In heterotransplants the crucial interplay of stroma-neoplasic components is recapitulated in a greater extent, contributing to better replication of pharmacogenomic profiles, histology, chromosome complement, antigen expression, and gene expression of human tumors, which accounts for their better predictive value as compared to cell line based xenografts. These models may also be used for molecular and histopathologic studies in order to identify key molecular markers involved in tumor response to chemotherapy.

However, there is also criticism associated with this model, related to the unrealistic growing conditions of the local environment, that results on rare development of metastasis, increased responsiveness to treatments and expression of different cell surface molecules [50].

The use of direct xenografting of human cancers allows the transplantation of all cellular fractions of the tumor, avoiding the clonal selection. Prior studies used different xenograft models to study the bladder carcinoma [60, 92, 1]. These models may provide a unique platform to test therapeutic approaches directed toward these cancers.

1.7 Aims of the project

Unpredictable outcomes from empiric therapy in patients with the same tumor type and stage is a widely recognized and frustrating problem for patients, their caregivers, and medical professionals. This is the case of invasive urothelial bladder cancer. Variations in the natural history and responses to treatment are seen between tumors with identical features, reflecting the heterogeneity of the constituent tumor cells. There has been a long-standing hope that new markers and approaches could be used to optimize therapy for each patient. To do that, we need to develop effective drug testing models, especially for invasive bladder tumors with higher risk of being chemo resistant.

The first goal of this project is to develop a direct xenograft model of invasive urothelial bladder carcinoma as a platform to predict tumoral sensitivity and resistance to cisplatin and gemcitabine *in vivo*. This study will be started at the time of patient cystectomy and have results in time to start the patient's chemotherapeutic regimen, if necessary. With the development of this model, we aim to select the best cancer treatment based on responsiveness of individual tumors and have the possibility of tailor treatment to individual patients—using effective agents while sparing unnecessary ones. The assessment of tumor sensitivity to chemotherapeutic drugs will be especially useful and justifiable for patients with higher risk of showing drug resistance. This risk will be evaluated through the expression of molecular markers, such as CD147, that have been associated with poor outcome and cisplatin resistance in previous studies.

The reproducibility of the model and maintenance of the phenotype of the original tumor will be assessed to guarantee the utility and validity of the model. This evaluation will be done through the analysis of the degree to which the mouse xenografts and patients' tumor correlate with each other in terms of histologic type and grade, expression of molecular markers of aggressiveness such as CD147, Ki-67, p53, p63 and Ck20.

The final goal of this project is to identify the profile of the patients that would benefit from this kind of drug testing and molecular markers associated with sensitivity or resistance to the main chemotherapeutic drugs used to treat invasive urothelial bladder carcinoma. We attempted to prospectively select individualized chemotherapy for patients based on the expression of tumor markers and in vivo assessment of drug sensitivity and resistance using xenografts established from fresh tumor samples.

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Chapter 2

Methods

2.1 Patient selection

The study consists of developing a direct invasive urothelial carcinoma xenograft model as a platform for predict tumor response to cisplatin and gemcitabine chemotherapy. Figure 2.1 shows a schematic representation of the study design.

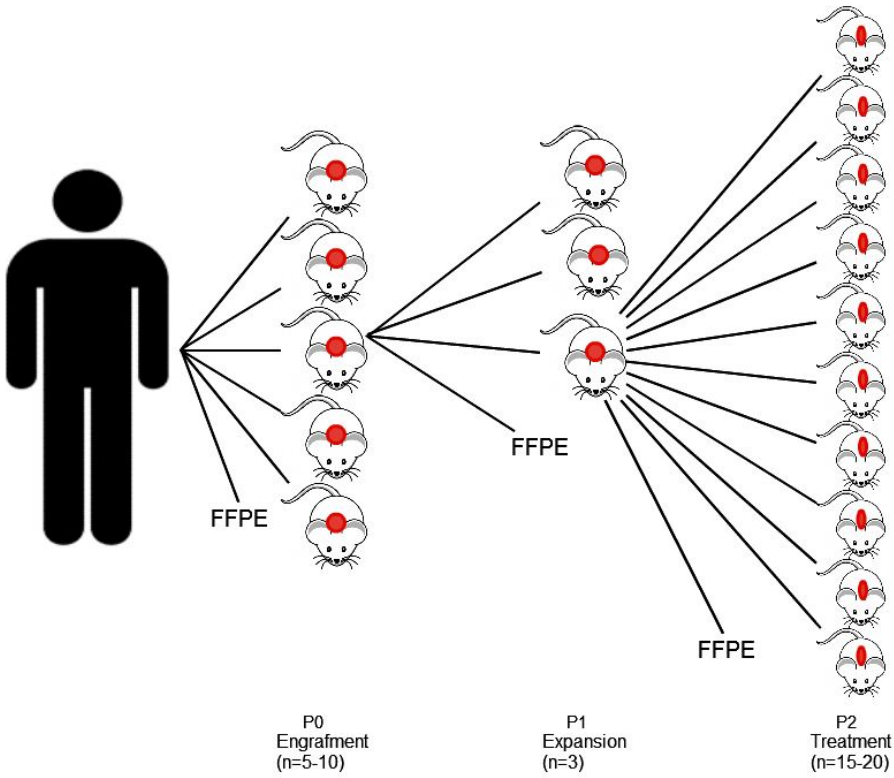


Figure 2.1: Schematic representation of the study design.

The patient selected to collect the sample for xenograft establishment met the following inclusion criteria: invasive urothelial carcinoma confirmed by histologic analysis, overexpression of emmprin, and therapeutic cystectomy with no previous neoadjuvant or adjuvant chemotherapy. Tumor sample for transplantation was obtained directly from the removed bladder and placed in RPMI medium with 1 % penicillin/streptomycin. Part of the sample was fixed in formalin for histologic and immunohistochemical analysis.

2.2 Animals

Immunodeficient mice bearing the nu/nu gene (strain: N:NIH(s) II-nu/nu) where obtained from the Animal Experimental Unit at IPATIMUP. These mice are characterized by thymic hypoplasia, depletion of T and B lymphocytes of both T-cell zones of lymph nodes and the spleen and poor development of hair. We used 9 males, 6 weeks old and a median weight of 17,6g.

2.3 Xenotransplant of tumor tissue

The tumor sample was cut into $1 - 2mm^3$ fragments and individual pieces of tumor were implanted subcutaneously (s.c.) through small horizontal incisions in the scapular regions of 9 anesthetized nude mice (P0 generation). The animals were anesthetized using isoflurane according to the manufacturer's instructions.

Following transplantation of tumor fragments, all mice were observed weekly. Tumor growth was assessed twice a week by measuring $length(d_1), width(d_2)$ and $height(d_3)$ with caliper, and estimated using the following formula:

$$TumorVolume = \frac{\pi}{6} \times d_1 \times d_2 \times d_3 \tag{2.1}$$

Tumor grwoth rate is characterized by the tumor volume doubling time (DT) which is the time period when $V_2 = 2V_1$ [70], and will be calculated using the following formula:

$$DT = (t2 - t1)ln2/ln(V_2/V_1) \tag{2.2}$$

2.4 Established cell line

During the development of direct xenografts of invasive urothelial carcinoma, an established cell line of urothelial carcinoma overexpressing emmprin was used to produce a tumor model *in vivo*. The cells grown in monolayer culture were harvested during the exponential growth phase using trypsin and suspended in medium plus. After quantification, the cells were re-suspended in medium without serum at a concentration of $1 \times 10^8 cells$ per mL of medium. Subcutaneous inoculation of the cells was done using $0,1mL$ of the cell suspension (1×10^7).

Monitorization and tumor volume assessment was carried out as described for the transplanted mice.

2.5 In vivo growth inhibition studies

Tumors were allowed to grow to a size of 1.5cm^3 at which point were harvested, divided, and transplanted to another mice (P1 generation). After second growth passage, tumors were excised and propagated to cohorts of >15 mice, which constituted the treatment cohort (P2 generation).

Tumors from P2 generation cohort were allowed to grow until reaching $200mm^3$, at which time they were uniformly distributed by size in the following groups: control injected with control vehicle, $0,2mL$ i.p. weekly for 5weeks (n=X), T1 injected with cisplatin, $3mg/kg$ i.p. weekly for 5 weeks (n=X) and T2 injected with gemcitabine, $20mg/kg$ i.p. weekly for 4 weeks (n=X). All mice were weighted and monitored for signs of toxicity, two times per week. Tumor size was evaluated twice a week by caliper measurements using the formula presented above. Relative tumor growth inhibition was calculated by relative tumor growth of treated mice, divided by relative tumor growth of control mice (T/C) since the initiation of therapy. The human end point criteria considered was weight loss > 30 % or debilitation.

The histology and immunohistochemistry of the original tumor and the tumors developed by the nude mice will be analyzed to characterize the tumors and evaluate the maintenance of the primary tumor phenotype.

2.6 Immunohistochemical analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue sections (3 μm) were tested with primary antibodies against CD147, p53, p63 Ki-67 and CK20 using polymer-HRP detection method (Power vision). Table **??** lists all antibodies, dilutions, incubation times and antigen-retrieval methods used. The sections were initially dewaxed in xylene and rehydrated in graded alcohols. Heat-induced epitope retrieval using citrate buffer was carried out according antibody manufacturer's instructions. Endogenous peroxidase activity was inhibited by immersing sections in H2O2(0.6 %) in distilled water for 20 min. Sections were rinsed in PBS-Tween prior to incubation with bovine serum albumin solution (20 min) to inhibit non-specific binding. PBS was subsequently used to wash sections between stages. Sections were then incubated with the primary antibodies against CD147, p53, p63 Ki-67 and CK20. The bound primary antibody was detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer (Power Vision poly-HRP-anti Ms/Rb/R IgG) for 30 minutes and DAB substrate for 7minutes. Then, the slides were counterstained with hematoxylin and mounted. Positive and negative controls was run simultaneously with tumor specimens. The staining patterns were assessed by two independent observers (one of them an histopathologist) using standard light microscopy.

Table 2.1: Overview of the antibodies used and tissue processing details.

Primary antibody	***Clone***	***Source***	***Dilution***	***Antigen retrieval***	***Incubation***
CD147	HIM6	Acris Antibodies (AM08346PU-N)	1:100	Citrate, pH 6.0, 20min	60min/room temp
P53	DO-7	Dako (M 7001)	1:100	Citrate, pH 6.0, 20min	Overnight/ 4°C
P63	4A4	NeoMarkers (MS-108-P1)	1:250	Citrate, pH 6.0, 20min	60min/room temp
Ki-67	DO-7	Dako (M 7240)	1:300	Citrate, pH 6.0, 20min	Overnight/ 4°C
CK20	Ks20.8	Novocastra (NCL-CK20)	1:250	Citrate, pH 6.0, 20min	Overnight/4°C

2.7 Histologic analysis

Tissue from the tumors and mice organs was fixed in 10 % phosphatebuffered formalin, embedded in paraffin, serially sectioned at 4 μm, and were stained with hematoxylin-eosin (H&E) for histological examination. The histologic type, degree of differentiation, nuclear aplasia and extension of invasion were registered for each tumor sample.

Chapter 3

Results

3.1 Pathologic features of the selected patient

The patient was a 69-year-old man with muscle invasive bladder cancer and was submitted to radical cystectomy with ilio-obturator lymph node dissection and uretero-ileal anastomosis according to the Bricker technique. The pathologic analysis revealed a unifocal solid nodular tumor ($3x2cm$) with surface ulceration located in the posterior wall of the bladder and diffuse carcinoma in situ. The histology showed a high grade urothelial carcinoma with muscularis propria and perivesical fat invasion (pT3a). There was also lymphovascular space invasion, but the lymph nodes (6) as well as the surgical margins were tumor free. In addition to the bladder carcinoma, foci of high grade prostatic intraepithelial neoplasia and in situ urothelial carcinoma in the prostatic urethra were also present.

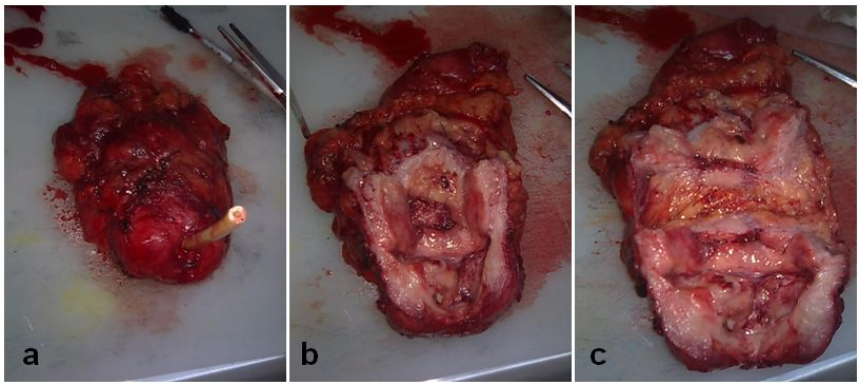


Figure 3.1: Radical cystectomy specimen showing an ulcerated lesion in the posterior wall of the urinary bladder.

3.2 Establishment of Xenografts

To date, 1 of 9 specimens has grown as primary implant in nude mice (11%, 1/9) as illustrated in the figure 3.2. At 6 weeks following transplantation one mouse (1a) was sacrificed to access if there was tumor growth in any part of the body. All organs were analyzed histologically and there was no evidence of tumor metastasis.

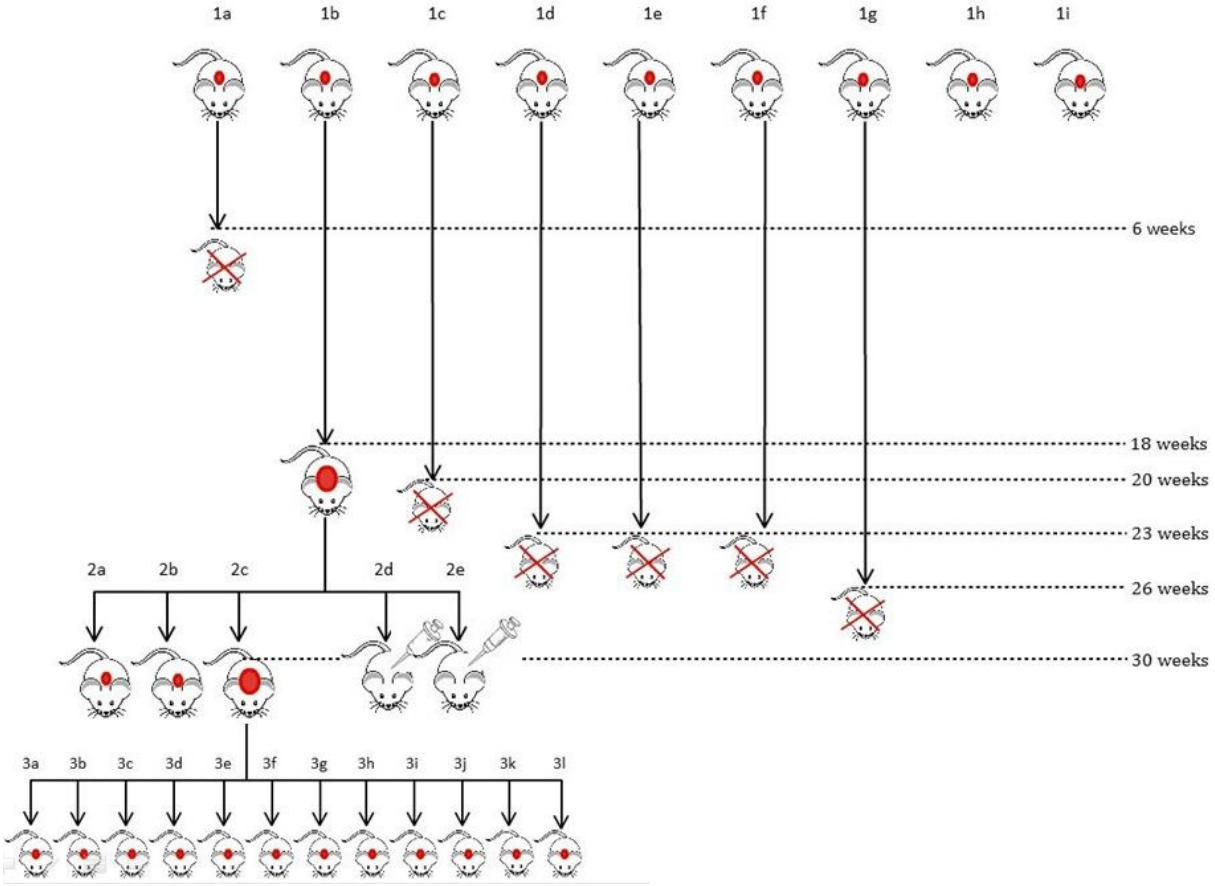


Figure 3.2: Schematic representation of mice evolution after tumor implantation. Tumor growth was seen in one mouse (1b) out of 9, and was excised at 18weeks after xetransplantation. This tumor was cut into fragments and implanted in 3 new mice (2a-c). The fluid of the cyst developed with the tumor was also removed and injected $(0,5ml)$ s.c. in 2 mice (2d and 2e). In the second passage, tumor growth was registered in 2 of the 3 mice, one of them (2c) was excised and transplanted to new mice (treatment group). After tumor excision, mouse 1b stayed alive for another 11 weeks, at that time, it was sacrificed due to severe weight loss (30%). The red cross represents euthanized mice. One mouse was sacrificed at 6 weeks to access if there was tumor growth in any part of the body. The other 5 mice were euthanized after showing severe weight loss.

The weight of the mice during the first part of the experience was similar between them and stable, however, some mice started to lose weight after 5 months as represented in the figure 3.3. Five recipient mice (1c, 1d, 1e, 1f and 1g) were euthanized after developing cachexia (at 20, 23 and 26 weeks) with weight losses above 30% and reduced mobility. Necropsy was performed on all euthanized animals. The histological analysis to the mice organs showed no evidence of tumor metastasis and the cause of weight loss remained unknown for four of them. The mouse sacrificed at 20 weeks (1c) showed hydronephrosis caused by a bladder stone. However, the reason for calculus formation remain unexplained.

Figure 3.3: Weight variation of the mice six months after xenotransplant.

3.2.1 Tumor growth

For the first week following implantation, a small bump was visible where the tumor was inserted and then it disappeared. At 13 weeks (3 months) after transplant, a mass began to appear at the site of implantation in one (mouse1b) of the 9 nude mice xenografted.

The xenograft had a lag period of 13 weeks before initial take was registered and achieved the exponential growth phase after the 18th week (see figure 3.4a). Over the period of 5 weeks, the tumor progressively grew to approximately $18mm$ in diameter (about $1,5cm^3$). The tumor volume doubling time was approximately 6 days. The xenograft grew as a solid subcutaneous tumor containing a fluid-filled sac. At the time of tumor excision no macroscopic evidence of local invasion was seen. The mouse bearing the tumor xenograft (1b) was sacrificed 11 weeks after tumor excision due to severe weight loss (30%). (Histologic analysis will be done in the next weeks..)

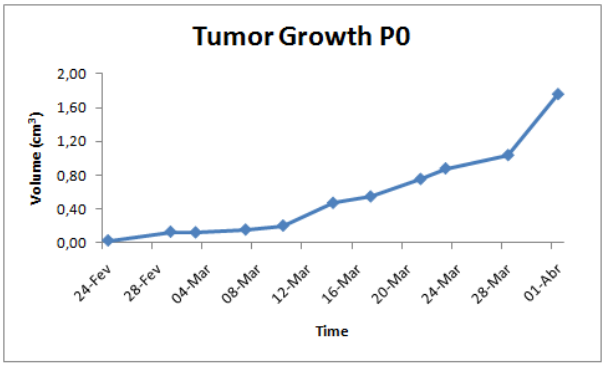
After excision, the human tumor xenograft was cut into fragments and implanted in 3 new mice (2a, 2b and 2c). The fluid of the cyst developed with the tumor was also removed and injected $(0,5ml)$ s.c. in 2 mice (2d and 2e). Tumor growth was seen in 2 (2d and 2c) of the 3 mice implanted at 5 and 9 weeks following transplantation, respectively. After 8 weeks, the tumor of the mouse 2c had reached approximately $2,5cm^3$ and was excised and passed to 12 mice (P2). The tumor developed was a solid subcutaneous tumor with no visible invasion of adjacent tissues. Figure 3.5a shows the tumor growth over the time. The tumor doubling time was approximately 8 days. The growth rate was constant over the time, with no exponential phase.

Tumor growth of the mouse 2b is represented in the figure 3.5b (green line), together with the tumor growth curves of 2a and 1b. This chart allows the comparison between the tumor growth rates of the P0 and P1 xenografts. This graphic shows a clear difference among the growth rate in the first and second passages. The xenograft P0 grew faster than the tumors P1, as suggested by the mean doubling time (6 and 8 days, respectively). To date, the growth rate of the tumor 2b is quite similar to the 2a, but the complete analysis of the growth pattern will only be possible when the tumor has reached the same level as the other two.

The two mice injected with the fluid provender from the cyst of the first xenograft did not showed any evidence of tumor growth until this moment.

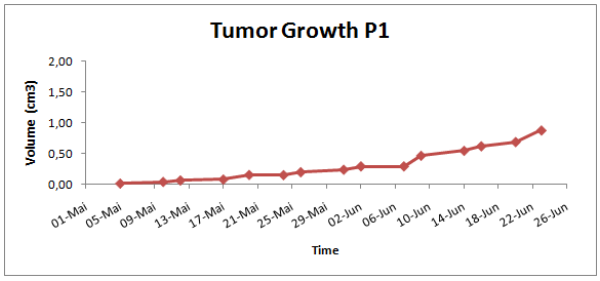
The injection of 1.5×10^6 MCR cell suspension in the subcutaneous space did not result in tumor establishment, so far (12 weeks after inoculation).

(a) Mouse 1b bearing a subcutaneous tumor developed after engraftment of human invasive urothelial bladder cancer (p0). Picture taken at the day tumor excision.

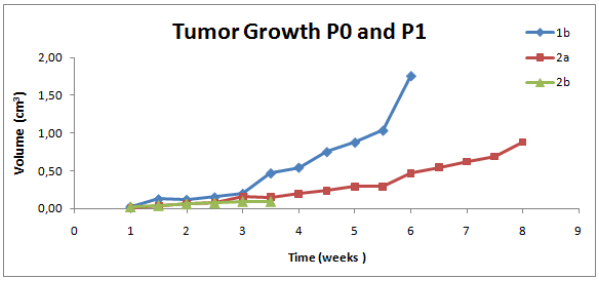


(b) Tumor growth curve of the P0 xenograft. Tumor take was registered 13 weeks after implantation.

Figure 3.4: Xenograft Establishment.



(a) Growth of human bladder tumor in nude mice, second passage. Tumor take was registered 5 weeks after implantation.



(b) Tumor growth: comparison between first (1b) and second passages (2a and 2b).

Figure 3.5: Tumor growth in nude mice.

3.3 H&E staining

3.3.1 Primary tumor histology

Histologically, the original tumor was an urothelial carcinoma with microscopic invasion of the muscularis propria layer and perivesical fat (pT3a). The malignant cellular features of this bladder tumor included high nuclear-to-cytoplasmic ratio, nuclear atypia and presence of mitotic figures. Figure 3.6 shows representative sections of the primary tumor stained with hematoxylin and eosin.

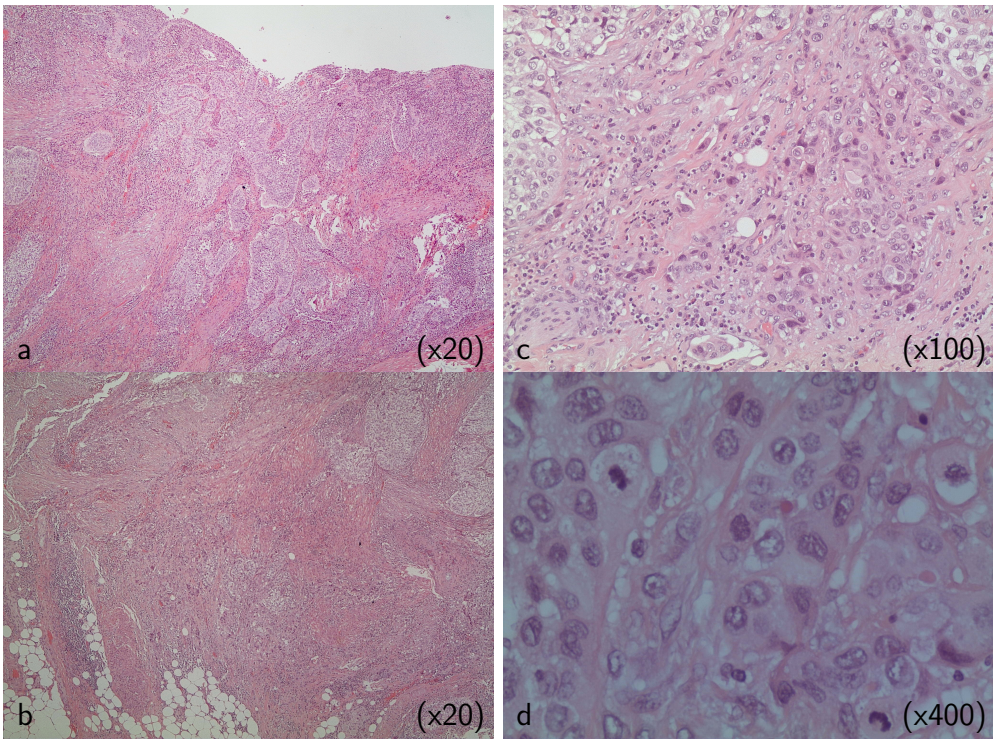


Figure 3.6: High grade invasive urothelial carcinoma showing nest of neoplasic cells infiltrating through the muscularis propria and perivesical fat of the bladder (a and b). Presence of lymphovascular space invasion (c) and atypical cells with high nuclear grade (nuclear atypia and hyperchromatic nuclei with marked anaplasia) and mitotic figures (d).

3.3.2 Xenograft histology

The histological analysis of the established xenograft revealed a tumor with cellular characteristics identical to those observed in the primary invasive urothelial tumor of the bladder as shown in the figure 3.7. In addition to the neoplasic cells, the xenograft also has a layer of epithelial like cells covering the tumor and in the wall of the cyst as shown in the figure 3.8. The vesicle-like sac is composed by an epithelial lining layer of variable number of cells and dense connective tissue.

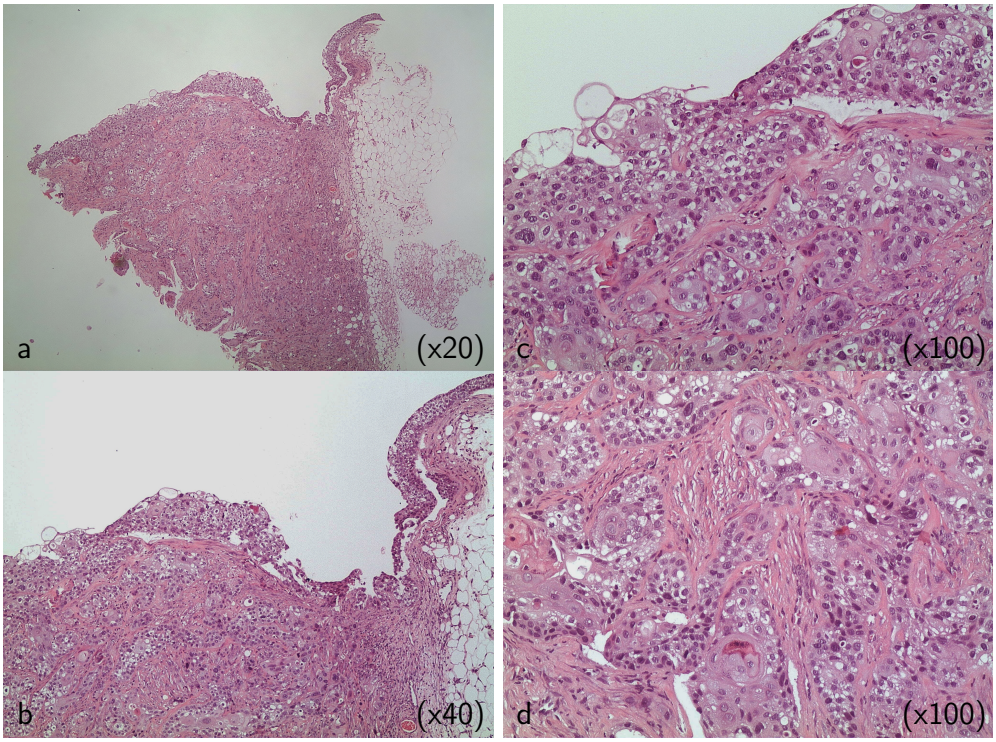


Figure 3.7: Micrograph of the tumor developed after xenograft, composed by nests of neoplasic cells (core) and desmoplastic stroma covered by a layer of epithelial cells (a and b). Neoplastic cells of moderate to large size with modest amounts of eosinophilic cytoplasm and nuclear atypia (b and c).

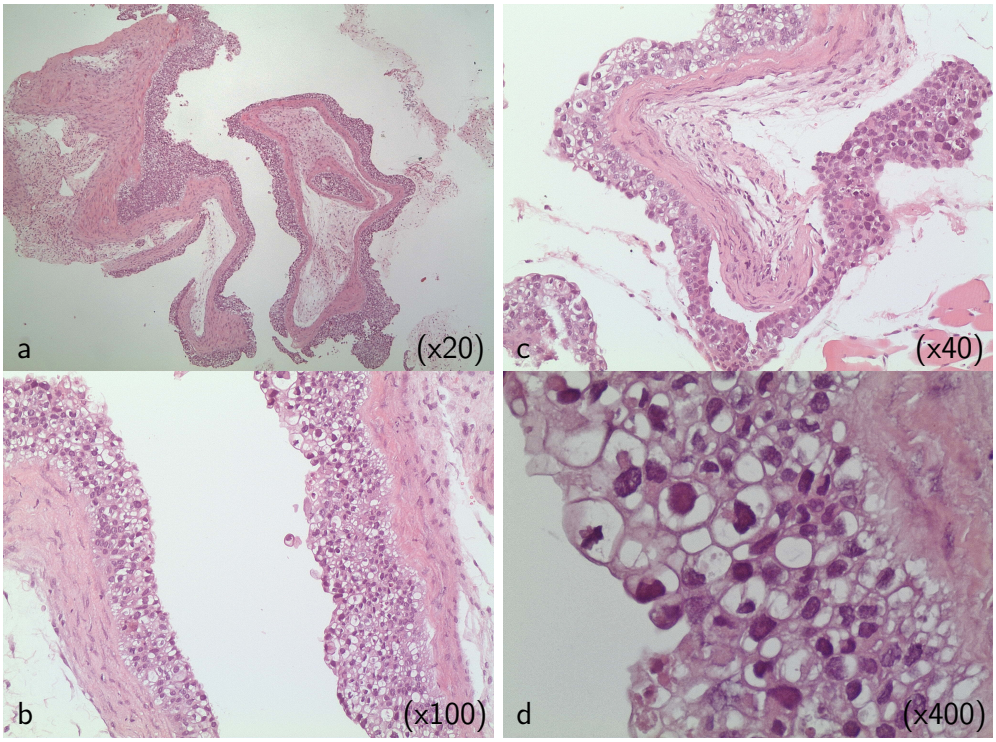


Figure 3.8: Micrographs of vesicle-like sac developed by the mouse after xenograft. This structure is composed by an epithelial lining layer of variable number of cells and dense connective tissue (a and b). The degree of nuclear atypia varies along the epithelial layer, having regions with large, hyperchromatic nuclei (c). The cells along the epithelial layer are bigger in the surface and have largest nuclei (d).

3.4 Immunohistochemistry

3.4.1 CD147 immunohistochemistry staining

The immunoreactivity to EMMPRIN or CD147 was observed in invasive urothelial bladder carcinoma cells of the primary tumor and in the tumor cells of the xenograft as shown in the figure 3.9. Positive immunoreaction was localized in the cytoplasm and membrane as it was expected due to the properties of the protein being analyzed.

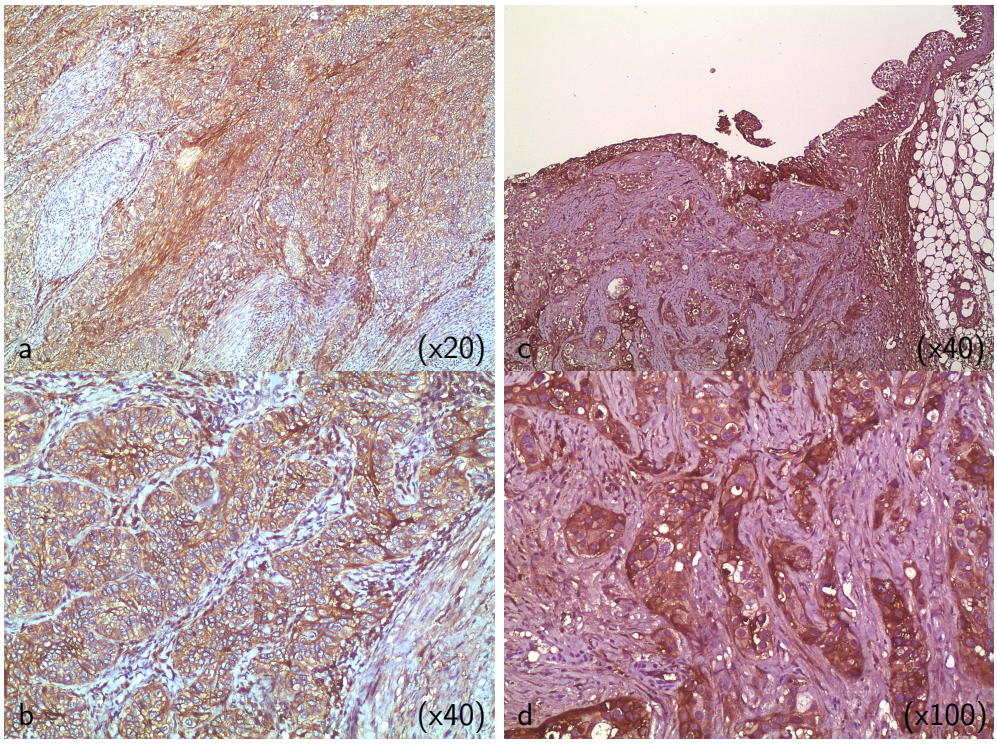


Figure 3.9: Primary invasive urothelial carcinoma (a and b) and xenograft (c and d) CD147 immunohistochemistry staining demonstrating citoplasmatic and membranar expression.

3.4.2 P53 immunohistochemistry staining

The protein p53 displayed nuclear expression in both primary and mouse tumor as shown in the figure 3.10. The primary urothelial carcinoma showed an heterogenic p53 staining pattern, having areas with more nuclear staining than the others. In the case of xenograft the percentage of stained nucleus was slightly higher than in the primary tumor, suggesting a more aggressive phenotype.

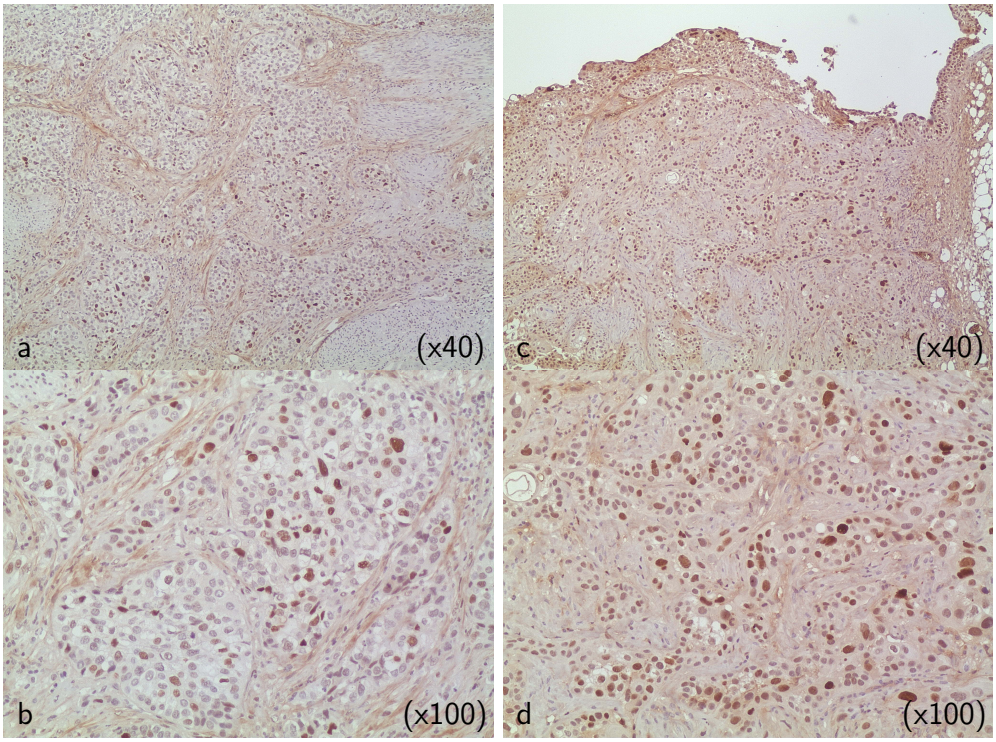


Figure 3.10: Primary invasive urothelial carcinoma (a and b) and xenograft (c and d) P53 immunohistochemistry staining demonstrating nuclear expression.

3.4.3 p63 immunohistochemistry staining

Expression of p63 in primary and mouse tumor specimen was present only in the neoplastic cells both in the primary tumor and in the xenograft as shown in the figure 3.11. Staining was always nuclear and were strong and diffuse both primary and mouse tumor. Although mouse tumor specimen showed some background staining, no staining was seen on smooth muscle cells, adipocytes or connective tissue.

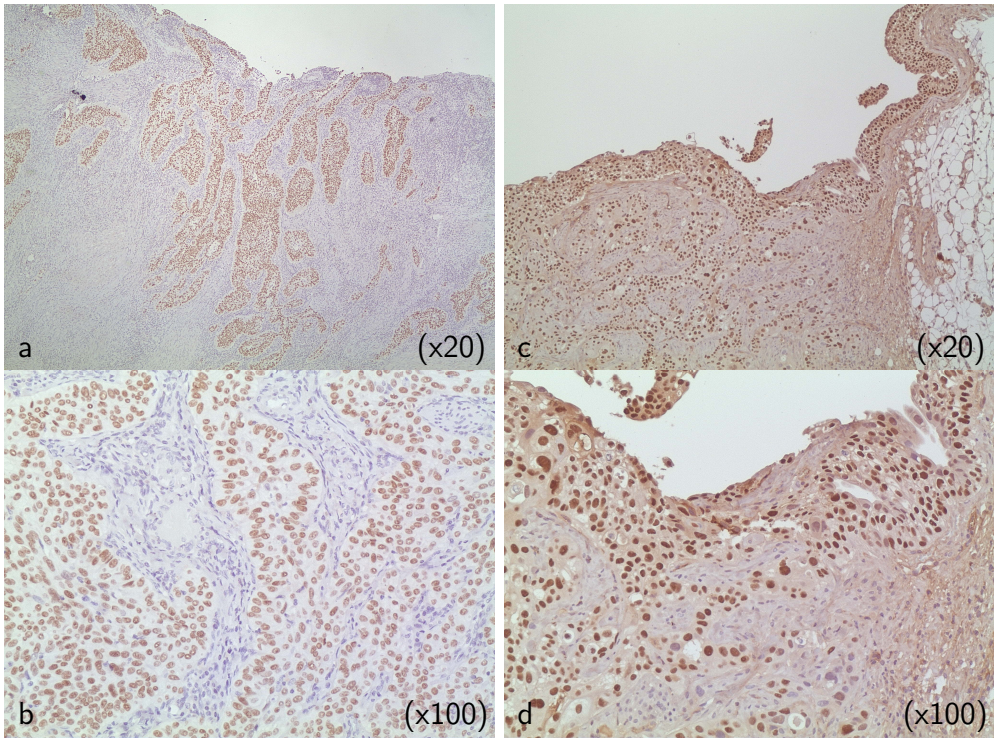


Figure 3.11: Primary invasive urothelial carcinoma (a and b) and xenograft (c and d) p63 immunohistochemistry staining demonstrating diffuse nuclear staining.

3.4.4 Ki 67 immunohistochemistry staining

Diffuse and heterogenic immunoexpression of Ki 67 was observed in the nucleus of both human invasive high-grade carcinoma and mouse tumor as shown in the figure 3.12. However the number of positive cells seemed slightly higher in the xenograft than in the primary tumor.

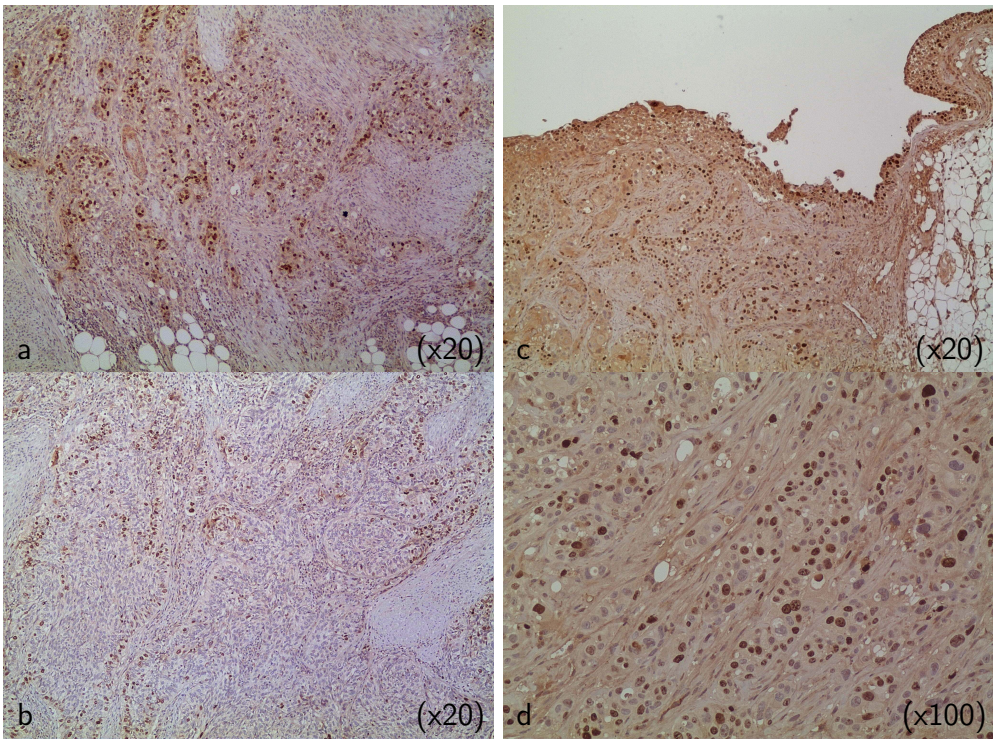


Figure 3.12: Primary invasive urothelial carcinoma (a and b) and xenograft (c and d) Ki67 immunohistochemistry staining demonstrating heterogenic and diffuse nuclear staining.

3.4.5 Citokeratin 20 immunohistochemistry staining

Citokeratin 20 expression has been described as an objective marker of the neoplastic change of urothelial cells. In the case of urothelial cells, the expression of CK20 is classified as normal when the expression is restricted to superficial (umbrella) cells and aberrant when there is cytoplasmic expression on urothelial cells, other than superficial umbrella cells. Both primary and mouse tumors showed an abnormal CK20 expression, being CK 20 positive in the full thickness of the tumor as shown in the figure 3.13.

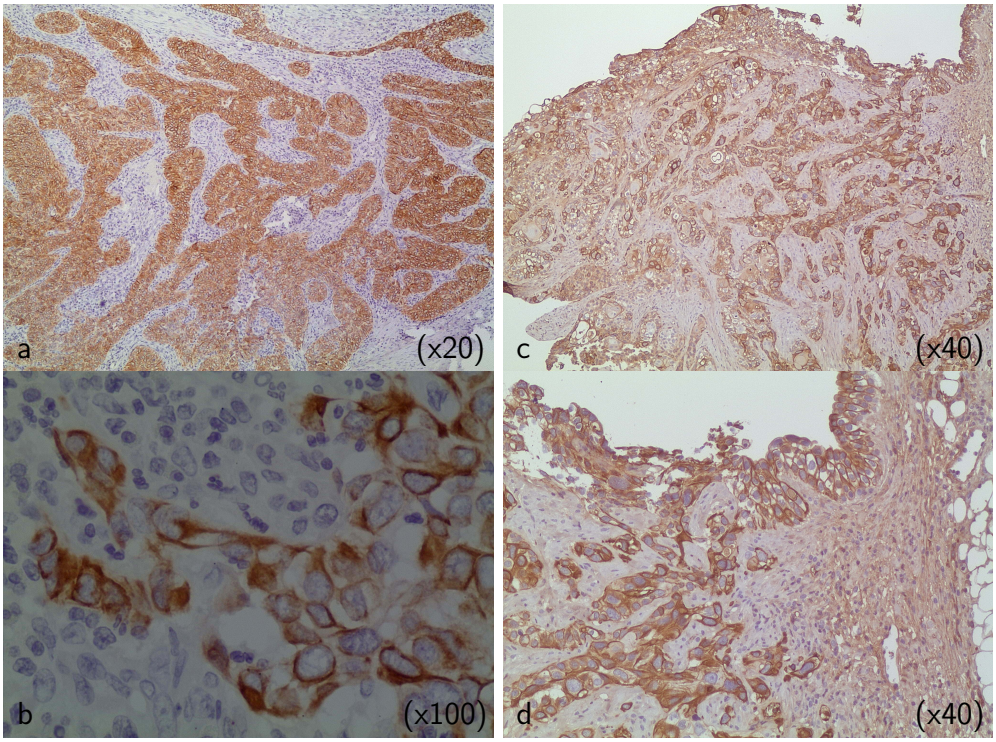


Figure 3.13: Primary invasive urothelial carcinoma (a and b) and xenograft citokeratin 20 immunohistochemitry staining showing difuse membranar/cytoplasmatic staining of tumor cell in the first case, and both tumor and epithelia cells in the second case (c and d).

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Chapter 4

Discussion

Unpredictable outcomes from empiric therapy in patients with invasive urothelial bladder cancer submitted to radical cystectomy is a widely recognized and frustrating problem for patients, their caregivers, and medical professionals. There has been a long-standing hope that new markers and approaches could be used to optimize therapy for each patient.

With this project we aimed to establish an urothelial cancer xenograft model in nude mice from a sample of invasive urothelial carcinoma, characterize it, and assess the feasibility of this model for chemotherapy sensitivity and resistance testing. By transferring fresh tumor fragments directly to nude mice, we successfully established 1 (11%) invasive urothelial cancer xenograft out of 9 trials. The resultant tumor consisted in a high grade solid tumor with cyst (fluid-filled sac) formation and cellular structure similar to the invasive urothelial carcinoma of the bladder of the donor patient. This xenograft was successfully passed to other mice (2/3), at least once.

Comparing with other studies, our success rate for xenograft establishment was lower [92]. Russell and colleagues reported that 11 of 20 specimens of bladder cancer have grown as primary implants in nude mice. The variation between success rates may be due to the different stage and grade of tumors implanted, the animal used or the volume of the fragment implanted ($1 \times 2mm^3$, in our case). Considering the volume of the fragment implanted, small tumor volume is considered better for obtaining necessary oxygen and nutrient supplies by passive diffusion before the acquisition of a new blood supply, however, the number of surviving cells needed for regrowth may be superior in large blocks than in small ones. The dimensions of the fragment may also influence the lag period.

The latency period (lag period), time between transplantation and first positive evidence of tumor growth, was 4 months for the first generation xenograft, and became shorter on the subsequent passage, 5 weeks. The long lag period observed for the first case might be explained by a low fraction of clonogenic cell present in the original tumor, and the need to adapt and growth in a new environment. Xenografts's growth rates were different between the first and second passage, however, until now, it was identical between the two xenografts in the second passage. Previous studies with human tumor mouse models reported occurrence of kinetic changes in the human tumor cells after transplantation and passage in the nude mice [99]. Most frequently, the transplanted tumor adapted to growth

in animals has a shorter doubling time than the original tumor isolated from a patient. Growth rates increase further during subsequent passages. The vascularity of the primary and transplanted tumor also differ, with transplanted tumors showing better blood supply and less necrosis. This difference could be due to selection of the most rapidly growing cells from a heterogeneous primary animal, secretion of paracrine growth factors (which induce neovascularization), or simply tumor size. The development of a fluid filled sac associated with the first xenograft may also contribute to the differences between growth rates. Volume change may be influenced by an increase in the fluid quantity, and not only by the tumor cell population, as it was expected. The consistency and reproducibility of tumor growth are important factors that will determine the utility of this model. This parameters will be carefully analyzed and compared in subsequent passages.

The tumor take rate and stability of the tumor in each passage are also important factors because it enables one to prepare sufficient numbers of xenografts to perform an assessment of chemotherapeutic drugs sensitivity and resistance. High tumor take rate and stability in passage will allow the correlation between growth delay after chemotherapy with the antitumoral activity of the drugs and exclude the influence of individual variance between xenografts.

There are many local factors that could influence cell growth and morphology of xenografts, including cell-to-cell and cell-to-matrix interactions, growth factors, cytokines and hormones. That is the reason, why it is important to perform drug testing only in the third tumor passage, allowing the substitution of the original tumor stroma, by host supporting cells. The microenvironment of the tumor–host interface is involved in many processes impacting on tumor development, including angiogenesis, growth, dissemination and metastasis of tumors. The crosstalk interaction between tumor cells and adjacent stromal cells participates in tumor immune escape, spreading and angiogenesis, which is conducted by a number of soluble and membrane molecules, including emmprin [118].

Histologic and immunohistochemical analysis suggest that xenografts retain their original morphology and phenotype, at least during xenograft establishment.

The consistency of the histological and immunohistochemical patterns seen in the original tumor and in the xenograft supports the value of this model for drug testing, however, comparison of this patterns with those of subsequent passages are required. If there is maintenance of morphologic and biochemical characteristics of the original tumor by the xenografts, it is expected that chemosensitivity would be similar in both the original and the xenografted human tumor, and that this correlation would be predictive for both active single agents and active drug combinations. Future results will give evidence concerning the local invasion and metastatic potential of tumor in mice.

In the present study, we examined the expression of CD147, P53, p63, KI-67, and CK20 in primary invasive urothelial carcinoma of the bladder and established xenograft. We found that CD147 was diffusely expressed in the cytoplasm and membranes of tumor cells both in the original and xenograft specimens. This molecular marker may be one of the major criteria to select patients that would benefit of the use of these animal model to access tumor sensitivity and resistance. CD147 expression has been described as a strong independent prognostic factor for response and survival after cisplatin-containing

chemotherapy in patients with advanced bladder cancer.

The other markers of proliferation(Ki-67) and aggressiveness (p53, p63 and CK20) analyzed were also highly expressed in both primary and xenograft tumors, with no significant differences between them. The slight increase in the expression of p53 and ki-67 seen in the xenograft when compared with the primary tumor may reflect a tendency to select of the most rapidly growing cells from a heterogeneous primary animal during engraftment. However, further studies are needed to evaluate if it is an isolated case or a tendency and if this alteration is statistically significant.

The cell line MCR failed to grow when inoculated (1×10^7) in the nude mice. It will be necessary to inoculate higher quantities of cells to access the tumorigenic potential of this cell line in nude mice.

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Chapter 5

Conclusion and future work

In conclusion, we were able to establish a direct human cancer xenograft model, that preserves the main biological characteristics of the original tumor, and may be useful to evaluate tumor sensitivity and resistance to chemotherapy in patients. The assessment of chemotherapy sensitivity and resistance of specific tumors will offer the potential of selecting cancer treatments based on responsiveness of individual tumors and use effective agents while sparing unnecessary ones, improving patient's outcome. However further studies will be necessary to optimize and validate this model. In terms of procedures, it will be necessary optimize standardize immunostaining reagents and techniques. The antibodies used for immunohistochemical analyzes were not validated for use in mouse specimens, as some of them have been obtained from mice. To overcome this limitation and validate the procedure, we made negative controls, however some background is still present.

In future work, it will be necessary to identify the profile of the patients that would benefit from this kind of drug testing. This objective can be achieved through the identification of molecular markers associated with sensitivity or resistance to the main chemotherapeutic drugs used to treat invasive urothelial bladder carcinoma, in the attempted to prospectively select individualized chemotherapy.

In the future, this model may also be used to develop preclinical studies with new drugs in order to identify new therapeutic protocols for resistant tumor to current drugs.

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